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Counteraction of *Bothrops* snake venoms by *Combretum leprosum* root extract and arjunolic acid

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ABSTRACT

Ethnopharmacological relevance: Serotherapy against snakebite is often unavailable in some regions over Brazil, where people make use of plants from folk medicine to deal with ophidic accidents. About 10% of *Combretum* species have some ethnopharmacological use, including treatment of snakebites.
Materials and methods: We evaluated the ability of the extract of *Combretum leprosum* and its component arjunolic acid to reduce some *in vivo* and *in vitro* effects of *Bothrops jararacussu* and *Bothrops jararaca* venoms. The protocols investigated include phospholipase, proteolytic, collagenase, hyaluronidase, procoagulant, hemorrhagic, edematogenic, myotoxic and lethal activities induced by these venoms in Swiss mice.
Results: Oral pre-treatment with arjunolic acid reduced the *Bothrops jararacussu* lethality in up to 75%, while preincubation prevented the death of all the animals. Hemoconcentration effect of *Bothrops jararacussu* venom was confirmed two hours after i.p. injection, while preincubation with arjunolic acid preserved the hematocrit levels. Both *Combretum leprosum* extract and arjunolic acid abolished the myotoxic action of *Bothrops jararacussu* venom. Preincubation of *Bothrops jararacussu* venom with the extract or arjunolic acid prevented the increase of plasma creatine kinase activity in mice. The hemorrhagic activity of *Bothrops jararaca* crude venom was reduced down to about 90% and completely inhibited by preincubation with 10 mg/kg or 100 mg/kg *Combretum leprosum* extract, respectively, while the preincubation and the pretreatment with 30 mg/kg of arjunolic acid reduced the venom hemorrhagic activity down to about 12% and 58%, respectively. The preincubation of the venom with both extract and 30 mg/kg arjunolic acid significantly reduced the bleeding amount induced by *Bothrops jararacussu* venom. The extract of *Combretum leprosum* decreased the edema formation induced by *Bothrops jararacussu* venom both in preincubation and pretreatment, but not in posttreatment. Similarly, arjunolic acid preincubated with the venom abolished edema formation, while pre- and posttreatment have been partially effective. Some enzymatic activities of *Bothrops jararacussu* and *Bothrops jararaca* venoms, *i.e.* phospholipase A_2, collagenase, proteolytic and hyaluronidase activities, were to some extent inhibited by the extract and arjunolic acid in a concentration-dependent manner.
Conclusions: Altogether, our results show that *Combretum leprosum* extract can inhibit different activities of two important Brazilian snake venoms, giving support for its popular use in folk medicine in the management of venomous snakebites.

1. Introduction

Medicinal plants have long been used in the treatment of snakebite, mainly in locations where it is difficult to obtain the specific antivenom. In some countries, knowledge and access to medicinal plant therapy is traditional, and such therapy can be important for many reasons. One is the lack of specific antivenom,

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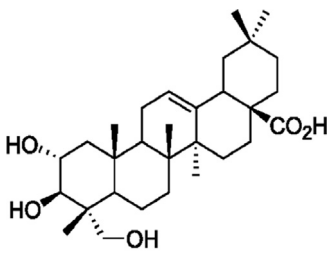
or because of the time needed to travel to a treatment location. Sometimes, therapy with specific antivenom may be partly or wholly ineffective (Mors et al., 1989, 2000a; Chippaux et al., 1991; Da Silva et al., 2007). These natural sources in folk medicine are important in many countries where snakebite treatment is a public-health problem, and its treatment is a challenge for these communities (Watt, 1989; Chippaux and Goyffon, 1997; Mors et al., 2000b; Gutiérrez et al., 2013a).

Although many species of plants are popularly known to be antiophidic agents, only a few have been investigated and had their active components isolated or characterized (Melo et al., 1994; Mors et al., 2000a; Coe and Anderson, 2005; Veronese et al., 2005; Strauch et al., 2013). Plants are important sources of bioactive components such as flavonoids, coumestans and triterpenes, which can help in the treatment of accidents with venomous animals (Mors et al., 1989, 2000a). Generally, substances of medical interest are found in very small quantities in the plants and are affected by many factors such as season of the year, time of collection, growth period, climate, soil composition, part of the plant from which the active component is extracted, or the batch collected (Havsteen, 1983; Mors et al., 2000a, 2000b).

In Brazil, the majority of accidents with venomous snakes are due to *Bothrops* sp. envenomation, which causes mainly local tissue damage, hemorrhage, edema and myonecrosis. Administration of the specific antivenom may prevent death but does not prevent local tissue damage and resultant disabilities (Da Silva et al., 2007). Antiophidic plants have been a target of previous studies from our group, such as *Eclipta prostrata*, which showed strong effects against venoms of snakes of the genera *Bothrops*, *Lachesis*, and *Crotalus* or some of their purified toxins, such as bothropstoxin, bothropasin, and crotoxin (Mors et al., 1989; Melo et al., 1994). The crude extract, as well as the isolated components wedelolactone, sitosterol, and stigmasterol, have shown intense antimyotoxic, antihemorrhagic, and antilethal activities against some snake venoms and isolated toxins (Mors et al., 1989; Melo et al., 1994; Melo and Ownby, 1999). Studies with *Casearia sylvestris*, *Harpalyce brasiliana* and *Humiriantherа ampla* have also demonstrated that these plants inhibited the myotoxic, edematogenic, and anticoagulant activities of several *Bothrops* venoms and isolated toxins (Borges et al., 2001; Da Silva et al., 2004; Strauch et al., 2013).

We have noticed that the plants from *Combretum* genus have been used as antivenom in folk medicine in Brazil and other regions of the world, especially in Africa. This plant is a member of the family Combretaceae, constituted by *circa* 600 species in 18 genera, of which *Teminalia* and *Combretum* are the most important. Worldwide, species of *Combretum* are popularly used against several diseases, most of the times used as infusions or decoctions of the leafs, flowers or roots (Facundo et al., 1993, 2005; Agra, 1996; Mors et al., 2000b; McGaw et al., 2001) including snakebites (Hutchings et al., 1996; Van Wyk et al., 1997). *Combretum leprosum* is found in Northeast Brazil, growing mainly along riverbanks, where it is called “mofumbo”, “mufumbo” or “pente de macaco” (Pio Corrêa, 1984). The phytochemical analysis of *Combretum leprosum* extract showed the presence of monosaccharides as the major compounds (80%), followed by triterpenes (10%) such as arjunolic acid (Fig. 1), and yet oligosaccharides (5%) and fatty acids (3%) (Facundo et al., 1993, 2005).



Arjunolic Acid

Fig. 1. The chemical structure of the pentacyclic triterpene arjunolic acid isolated from *Combretum leprosum*.

In the present study, the *Combretum leprosum* extract and the isolated triterpene arjunolic acid were investigated under different experimental protocols *in vivo* and *in vitro* against some important activities of *Bothrops jararacussu* and *Bothrops jararaca* venoms.

2. Material and methods

Bothrops jararaca and *Bothrops jararacussu* venoms and the antibothropic polyvalent antivenom (PAV) were obtained from Instituto Vital Brazil, Niterói, RJ. Bothropstoxin II was obtained from Faculdade de Ciências Farmacêuticas/USP, Ribeirão Preto-SP. Creatine kinase (CK) activity was determined using a CK NAC® kit from BIOCLIN®. Azocasein®, azocoll®, and hyaluronic acid were purchased from Sigma Chemical Co., USA. Male Swiss mice were provided by the Rodent Vivarium of the Institute of Microbiology Paulo de Góes – Federal University of Rio de Janeiro. Mice (25.0 ± 1.0 g) used for the study received water and food *ad libitum* and were kept under a natural light cycle. We adhered to protocols approved by the Ethics Committee for the Use of Animals of the Federal University of Rio de Janeiro (CEUA-UFRJ).

2.1. Plant material, extraction and isolation

Botanical material (*Combretum leprosum*) was collected in May 2001 in Viçosa, State of Ceará, Brazil. The material was identified at the Prisco Bezerra herbarium of the Biology Department, Federal University of Ceará, and a voucher specimen was deposited under number 12446.

Ethanolic extract was obtained from the roots (2.7 kg), which were placed in contact with ethanol 98% (5 L) at room temperature for 72 h. The material was filtered and the solvent evaporated under reduced pressure, yielding 58.3 g of extract. The isolation of arjunolic acid was performed as previously described (Facundo et al., 1993). Part of the extract (34.0 g) was submitted to coarse chromatography over silica gel using as eluents hexane, clorofórmium, ethyl acetate, and methanol. In the fraction eluted with ethyl acetate the presence of a white precipitate was observed, which, through recrystallization in methanol, was identified as the triterpene arjunolic acid. Spectral data, mainly mass spectrometry, ^{1}H and ^{13}C NMR were in agreement with literature values (Facundo et al., 1993).

2.2. Myotoxicity in vivo and in vitro

We evaluated the *in vivo* myotoxicity of *Bothrops jararacussu* venom by measuring the increase of plasma CK activity induced by perimuscular injection of venom alone or associated with *Combretum leprosum* extract or arjunolic acid. The venom was dissolved in physiological saline solution (PSS) to a final volume of 0.1 mL (1.0 mg/kg) and injected next to the *extensor digitorum longus* (EDL) muscles, in order not to cause direct mechanical damage to the muscle, as previously described (Calil-Elias et al., 2002b). Negative controls consisted of mice injected with the same volume of PSS. To evaluate the antimyotoxic activity of the *Combretum leprosum* extract (250 mg/kg) and arjunolic acid (70 mg/kg), three different protocols were used, as follows. Pre-incubation: venom dissolved in PSS was first incubated with the extract or arjunolic acid for 30 min at room temperature prior to injection; pretreatment: 0.15 mL of the extract or arjunolic acid was injected intraperitoneously (i.p.) 15 min before the i.m. venom injection; post-treatment: 0.15 mL of the extract or arjunolic acid

was injected i.p. 15 min after the i.m. venom injection. Blood was collected under anesthesia from the orbital plexus with heparinized capillaries, immediately before and two hours after venom injection, and CK activity in plasma was determined according to previously described procedures (Melo and Suarez-Kurtz, 1988a, 1988b). Twenty-four hours after venom injection, mice were sacrificed under anesthesia and had their EDL muscles isolated to measure CK content within the muscles. The doses used here were based on the dose-response study (not shown), from where we chose the most effective ones.

In order to assess the loss of creatine kinase from isolated muscle cells, which indicates damage to the sarcolemma, *in vitro* assays were performed as previously described (Melo and Suarez-Kurtz, 1988b; Melo et al., 1993, 1994). Briefly, mouse *extensor digitorum longus* (EDL) muscle was removed, mounted in collecting units and continuously superfused, using a peristaltic pump, with physiological saline solution (PSS), which was composed of (mM): NaCl, 135; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; $NaHPO_4$, 1; $NaHCO_3$, 15; and dextrose, 11. The pH of this solution was equilibrated to 7.3 with 5% CO_2 / 95% O_2. During the superfusion, the muscles were exposed to *Bothrops jararacussu* venom (25 μg/mL) or Triton-X (0.01%), and *Combretum leprosum* extract (10–100 μg/mL) or arjunolic acid (10 and 100 μg/mL) that were added to the PSS. Perfusion samples were collected at 30 min intervals and replaced with fresh solution. The collected samples were stored at 4 °C and their CK activity was determined with uv spectrophotometry at 340 nm, according to previously described procedures, considering the muscles' masses (Melo and Suarez-Kurtz, 1988a, 1988b).

2.3. Thigh edema

The induction of edema was evaluated by an intramuscular injection of 50 μL *Bothrops jararacussu* venom (1 mg/kg). Three treatment protocols were used: the venom was preincubated at room temperature with the crude extract (100 mg/kg) or arjunolic acid (30 mg/kg) for 30 min before the i.m. injection; the extract (100 mg/kg) or arjunolic acid (30 mg/kg) was orally administered 15 min before the venom injection; and the post-treatment, where the animals received the extract (100 mg/kg) or arjunolic acid (30 mg/kg) i.p. injection 5 min after the venom injection. The doses used here were based on the dose-response study (not shown), from where we chose the most effective ones. The thigh area was measured using a caliper rule.

2.4. Hemorrhagic activity

The hemorrhagic effect was induced by an intradermic (i.d.) injection of 0.1 mL of *Bothrops jararaca* venom (1 mg/kg) in the abdomen of mice and quantified as previously described (Melo et al., 1994). The negative control received 0.1 mL of PSS i.d. injection. To evaluate the antihemorrhagic activity of the *Combretum leprosum* extract and arjunolic acid, three different protocols were used: preincubation: *Bothrops jararaca* venom dissolved in PSS was first incubated with the extract (10 or 100 mg/kg) or arjunolic acid (3 and 30 mg/kg) for 15 min at room temperature prior to injection; pretreatment: 0.2 mL of arjunolic acid (3 and 30 mg/kg) was administered orally 30 min before the intradermic (i.d.) venom injection; post-treatment: 0.15 mL of arjunolic acid (30 mg/mL) was administered i.p. 5 min after the intradermic (i.d.) venom injection. Two hours after the venom injection, animals were killed by ether anesthesia, and the skin covering the abdomen was removed, stretched, and dried at room temperature for 72 h. The skin was fixed to a lucite base plate, and the entire area at the injection site and the surrounding area were transilluminated using an incandescent light. Light transmitted over an area of 109 mm^2 was read, and light transmission or the absorbance was normalized by taking the mean values of the readings over skin injected with either PSS (calibrated to zero) or *Bothrops jararaca* venom as arbitrary units of absorbance (AU). The doses used here were based on the dose-response study (not shown), from where we chose the most effective ones.

2.5. Tail bleeding evaluation

Tail bleeding tests were performed in groups of 5 mice each by a modified method, as previously described (Broze et al., 2001). *Bothrops jararaca* venom was administered (0.1 mg/kg, i.v.) in anesthetized mice (pentobarbital 40 mg/kg i.m.+xylazine 2.5 mg/kg i.p.). Several minutes later, the mice were immobilized in a restraint device and had their tails cut 4 mm from the tip and immersed in tubes with 5.0 mL of normal distilled water at room temperature. Blood loss was evaluated one hour later as a function of absorbance at 540 nm due to the hemoglobin content in the saline solution. The absorbance detected for a group that received saline rather than venom was taken as a control of blood loss, and the amount of bleeding in each procedure was expressed as units of absorbance in 540 nm. For evaluation of the antiophidic activity, the venom was preincubated at room temperature with *Combretum leprosum* extract (30 mg/kg) or arjunolic acid (1–30 mg/kg) 30 min before the i.v. injection, and a group of animals received oral pretreatment with arjunolic acid (30 mg/kg) 30 min before venom injection.

2.6. Clotting time

The clotting time was assessed by the modified Lee–White method (Raphael, 1983). The animals were grouped ($n=4$ for each group) and about 50 μL of blood were collected from the orbital plexus using microhematocrit capillary tubes. Before collecting blood, the tubes were filled with 20 μL of PSS; *Bothrops jararacussu* or *Bothrops jararaca* crude venoms (1 μg/mL); and venom preincubated for 30 min with *Combretum leprosum* extract (1 and 10 μg/mL) or arjunolic acid (1 and 10 μg/mL). The clotting time was evaluated and compared among the different groups.

2.7. Phospholipase activity

Phospholipase A_2 activity was assessed by adapting the turbidimetric assay described previously (Marinetti, 1965). We prepared the substrate by shaking one chicken egg yolk in a solution of 150 mM NaCl to a final volume of 100 mL and stored this at 4 °C prior to the reaction. In each assay, we prepared several tubes by taking a final volume (0.33 mL) of a 10% dilution of the egg yolk suspension and adding it to a solution containing 150 mM NaCl, 10 mM $CaCl_2$, 0.01% taurocholic acid, and 5.0 mM Tris–HCl (pH 7.4), The tubes were kept at 37 °C during the procedure. The reactions were started by adding 10 μg/mL of *Bothrops jararacussu* or *Bothrops jararaca* venom or 3 μg/mL bothropstoxin II (BthTx-II, a phospholipase A_2 from *Bothrops jararacussu* venom) alone or preincubated for 30 min at 37 °C with *Combretum leprosum* extract (10–100 μg/mL) or arjunolic acid (0.1–300 μg/mL). Thirty minutes after starting the reactions, the absorbance of the solutions was read at 925 nm, and data expressed as turbidity decrease and venom activity (%).

2.8. Proteolytic activities

Azocasein and azocoll were used to determine the proteolytic activities of *Bothrops jararaca* and *Bothrops jararacussu* venoms and to assess the antagonism by *Combretum leprosum* extract and arjunolic acid.

The proteolytic assay was carried out as previously described (Garcia et al., 1978). *Bothrops jararacussu* and *Bothrops jararaca* venoms (10 μg/mL) were preincubated with *Combretum leprosum* extract (0.1–300 μg/mL) or arjunolic acid (1–300 μg/mL) for 30 min at 37 °C. We added the venoms alone or the mixtures in a solution containing 0.2% azocasein, 20 mM $CaCl_2$, and 0.2 M Tris–HCl (pH 8.8). The reaction run for 90 min at 37 °C and was stopped by the addition of 0.4 mL of 15% (vv) trichloracetic acid, and then centrifuged at 20,000 rpm. We then removed 1.0 mL of the supernatant and mixed it with 0.5 mL NaOH (2.0 M). This final solution was analyzed by spectrophotometry at an absorbance of 420 nm.

The collagenase activity was assessed by the modified method of Chavira et al. (1984). Azocoll (0.2 mL, 0.15%), used as substrate, was suspended in Tris–HCl buffer solution pH 8.0 (0.2 M, 20 mM $CaCl_2$) and further added to 0.4 mL of Tris–HCl solution pH 7.5 (5.0 mM). The venoms of *Bothrops jararacussu* and *Bothrops jararaca* (50 μg/mL), alone or in the presence of *Combretum leprosum* extract (1.0–300 μg/mL) or arjunolic acid (1.0–100 μg/mL), were dissolved in azocoll solution. The reaction run for 90 min at 37 °C and was then centrifuged. Then, the supernatant (0.4 mL) was collected, 0.4 mL of 15% trichloroacetic acid was added to stop the reaction, and the resulting solution was analyzed by spectrophotometry at an absorbance of 520 nm.

2.9. Hyaluronidase activity

We used the method described by Di Ferrante (1956), with minor modifications, to assess the ability of *Bothrops jararacussu* and *Bothrops jararaca* venoms (50 μg/mL) to degrade hyaluronic acid. The hyaluronic acid solution (10 μg/mL) was prepared in acetate buffer solution (0.02 M, pH 6.0). The venoms alone or pre-incubated with arjunolic acid (0.1–30 μg/mL) for 30 min were added to the solution, and the tubes were incubated for 15 min at 37 °C. Hexadecytrimethylammonium bromide (0.666 mL, 2.5%) was added to the tubes for absorbance readings at 400 nm. Results are expressed in percentage of venom activity *vs.* arjunolic acid concentration.

2.10. Systemic effects (lethality and hematocrit)

In order to evaluate the systemic effect of *Bothrops jararacussu* venom and the protection by arjunolic acid, mice (groups of 5) received intraperitoneal (i.p.) injection of venom (5 mg/kg). The triterpene was given orally (30 and 100 mg/kg) 30 min before venom (pre-treatment); together with the venom following 30 min preincubation (100 mg/kg); or 5 min after venom injection (100 mg/kg, posttreatment). Mice were observed during 72 h to quantify the number of survivors.

Another set of animals also received intraperitoneal (i.p.) injection of *Bothrops jararacussu* venom (5 mg/kg), plus oral pre- and posttreatment with arjunolic acid (30 mg/kg) 30 min before and 5 min after venom injection, respectively, besides injection together with the venom following 30 min preincubation (30 mg/kg). In all groups, the animals were anesthetized under diethyl ether for blood collection 2 h after venom injections to evaluate the hematocrit. The blood was collected from the orbital plexus with heparinized capillaries, centrifuged and analyzed using a percentage hematocrit ruler.

2.11. Statistical analysis

Data were expressed as mean ± SEM, and One-Way Analysis of Variance (ANOVA) was used to compare groups with one variable, followed by Dunnett's post-hoc test. For two variables the Two-Way Analysis of Variance (ANOVA) was used followed by Bonferroni's post-hoc test. The p value < 0.05 was used to indicate a significant difference between means. The software GrapPad Prism version 5.01 was used to provide statistical analysis.

3. Results

3.1. Myotoxic activity

The venom of *Bothrops jararacussu* showed myotoxic activity, confirming previous observations. Mice isolated EDL muscles exposed to PSS showed a basal rate of CK release of 0.52 ± 0.07 U.g^{-1}.h^{-1} that increased up to 50.38 ± 3.47 U g^{-1} h^{-1} when exposed during 90 min to *Bothrops jararacussu* crude venom (25 μg/mL, $n=4$). Perfusion in the presence of the crude extract of *Combretum leprosum* (10 and 100 μg/mL) reduced the rate of CK release (33.13 ± 2.08 and 0.20 ± 0.12 U g^{-1} h^{-1}, respectively, $n=4$) (Fig. 2A). Thereafter, two groups of EDL muscles were perfused during 120 min. One of the groups was bathed with venom alone (25 μg/mL) for the first 60 min and then with venom+extract (100 μg/mL) for the remaining 60 min, while another group of muscles received venom+extract until 60 min, when extract was removed. Interestingly, the profile of CK release changed in the two groups, as we can observe in Fig. 2B.

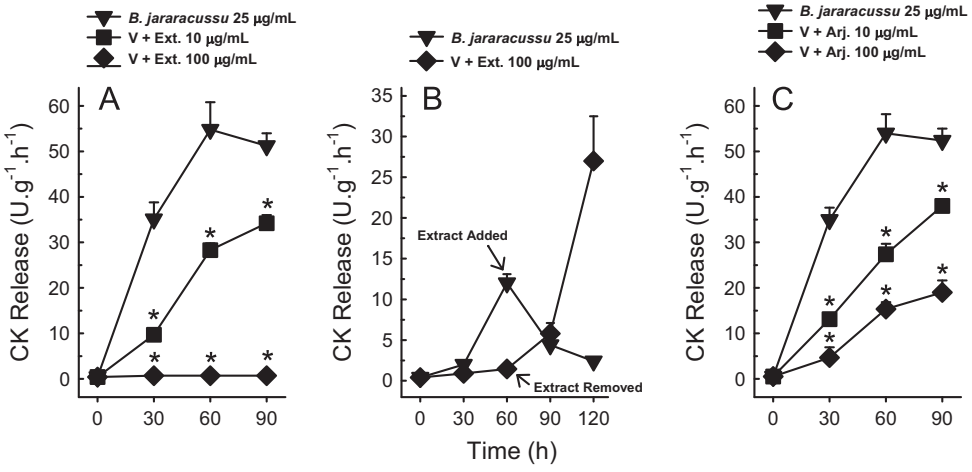


Fig. 2. Effect of *Combretum leprosum* extract and arjunolic acid on *Bothrops jararacussu* venom myotoxicity *in vitro*. The panels show the rate of creatine kinase (CK) release from isolated mouse *extensor digitorum longus* (EDL) muscles following exposure to *Bothrops jararacussu* venom (V) alone or incubated with *Combretum leprosum extract* (Ext., panel A) or arjunolic acid (Arj., panel C). Panel B shows the effect of changing the bathing solutions (venom alone and venom plus extract). Data report mean ± SEM ($n=4$). *$p < 0.05$ for the difference between treated groups and the venom. Two-Way ANOVA Bonferroni's post-hoc test.

On its turn, arjunolic acid (10 and 100 μg/mL) also reduced the rate of CK release (38.0 ± 1.34 and 19.0 ± 2.64 U g^{-1} h^{-1}, respectively) (Fig. 2C). Finally, we tested the myotoxic activity of the unspecific cytotoxic agent Triton-X. When exposed to Triton-X alone (0.01%), the muscles' rate of CK release reached 33.77 ± 4.76 U g^{-1} h^{-1}, which did not change significantly in the presence of *Combretum leprosum* extract (28.19 ± 4.19 U g^{-1} h^{-1}), showing that the extract does not interfere with the type of cellular injury caused by Triton-X (data not shown).

Intramuscular injection of *Bothrops jararacussu* crude venom (1 mg/kg) caused an increase in plasma CK activity compared to PSS injection (maximum activities two hours after injection: 2170.41 ± 287.35 U/L *vs.* 62.96 ± 5.07 U/L respectively, $n=5$). Neither the pre- nor the posttreatment with *Combretum leprosum* extract (250 mg/kg) were able to alter the venom myotoxic activity (1933.27 ± 316.01 and 2156.48 ± 187.40 respectively), while pre-incubation of the venom with the same dose of *Combretum leprosum* extract significantly inhibited the venom activity (285.36 ± 78.95 U/L; Fig. 3A). On the other hand, when we tested arjunolic acid instead of crude extract we also observed preincubation inhibiting *Bothrops jararacussu* myotoxicity, but here the pre-treatment also showed some inhibition (Fig. 3B). Arjunolic acid was also effective in preserving CK content within EDL muscle 24 h after perimuscular venom injection comparing to PSS injection (venom: 516.98 ± 46.00 and PSS: 819.58 ± 35.76 U/g), especially in pre-incubation protocol (770.00 ± 47.00 U/g) (Fig. 3C).

3.2. Edematogenic activity

Bothrops jararacussu venom i.m. injection (1.0 mg/kg) led to edema formation in mice hind limb, which was measured during 90 min with a peak of edema in around 15 min (Fig. 4A and C). Fig. 4B and D shows the area under the curve calculated from Fig. 4A and C. We can seen in Fig. 4B that the venom increased the AUC in about 42% compared to PSS injection. *Combretum leprosum* extract, both in preincubation and pretreatment, but not in posttreatment, decreased the edema formation. Similarly, arjunolic acid preincubated with the venom abolished edema formation, while pre- and posttreatment have been partially effective (Fig. 4D).

The loss of fluid to the third space, *i.e.* to the interstitium, leading to edema formation, was also observed indirectly by measuring the hematocrit. Hemoconcentration caused by of *Bothrops jararacussu* venom was confirmed two hours after intraperitoneal injection (data not shown), when hematocrit levels reached $55.2 \pm 0.9\%$, while animals receiving PSS presented $48.8 \pm 1.5\%$. Preincubation with arjunolic acid 30 mg/kg maintained the hematocrit levels similar to PSS ($48.9 \pm 0.8\%$), while pre-treatment showed partial effect ($51.0 \pm 0.4\%$) and posttreatment had no effect.

3.3. Effect of Combretum leprosum extract and arjunolic acid on hemostasis

The intradermic injection of *Bothrops jararaca* crude venom (1 mg/kg) induced intense hemorrhage on mouse abdomen skin when compared to animals receiving only PSS injection (from 226.62 ± 56.23 to 591.12 ± 60.00 arbitrary units), and was used as 100% of hemorrhagic activity. When the venom was preincubated with 100 mg/kg *Combretum leprosum* extract the hemorrhagic activity was completely inhibited. Posttreatment of the animals with arjunolic acid did not prevent the skin hemorrhage (not shown), but the preincubation and the pretreatment with 30 mg/kg of arjunolic acid reduced the venom hemorrhagic activity down to about 12% and 58%, respectively (Fig. 5A).

Bothrops jararaca venom (0.1 mg/kg) was able to induce an increase in the bleeding amount from mice tails, from 410.60 ± 33.35 (PSS control) up to 831.40 ± 83.44 absorbance units at 540 nm (Fig. 5B). The preincubation of the venom with both the extract and arjunolic acid (30 mg/kg) reduced the bleeding amount down to 657.00 ± 38.00 and 407.00 ± 40.00 absorbance units, respectively, but the oral pretreatment was not effective (not shown).

The clotting time when microhematocrit capillary tubes were prefilled with PSS was 157.44 ± 11.32 s (Fig. 5C). *Bothrops jararaca* venom induced a procoagulant activity, reducing the clotting time of mouse blood down to 18.14 ± 2.47 s. *Combretum leprosum* extract at 10 μg/μL completely inhibited the procoagulant activity induced by *Bothrops jararaca* venom (181.25 ± 37.78 s). Arjunolic acid (10 μg/mL) also inhibited *Bothrops jararaca* activity (140.00 ± 10.00 s), and interestingly the concentration of 30 μg/mL prevented blood coagulation for more than 10 min (741.60 ± 40.98 s, data not shown). *Bothrops jararacussu* venom also presented procoagulant activity, with the same profile of inhibition by *Combretum leprosum* extract.

3.4. Enzymatic activities

3.4.1. Phospholipase activity

The phospholipases present in the venoms of snakes *Bothrops jararacussu* and *Bothrops jararaca,* when placed in contact with a phospholipid substrate, such as lecithin (phosphatidylcholine), decreased the turbidity of the egg yolk solution used in the experimental protocol. The venoms and BthTx-II caused a concentration-

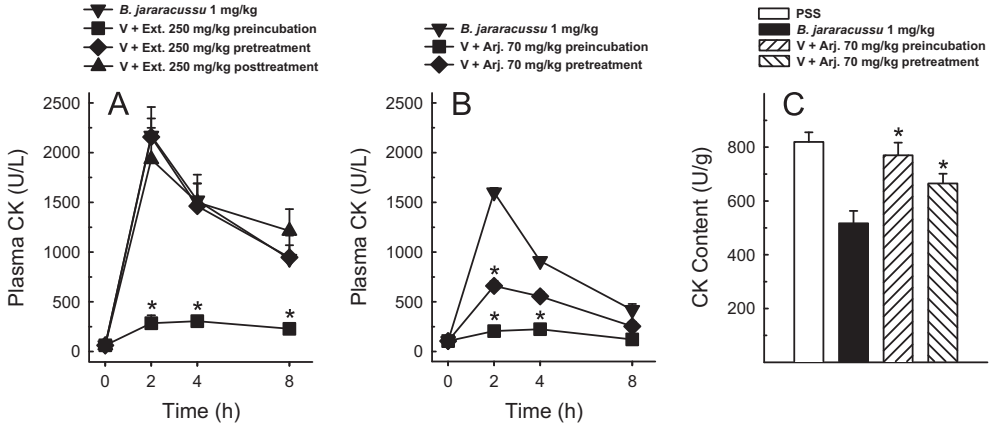


Fig. 3. Effect of *Combretum leprosum* extract and arjunolic acid on *Bothrops jararacussu* venom myotoxicity *in vivo*. Mice received i.m. injection (0.1 mL) of *Bothrops jararacussu* venom (V) alone or associated with *Combretum leprosum* extract (Ext., panel A) or arjunolic acid (Arj., panel B) in different protocols. Results show plasma CK activity 2 h after venom injection. Panel C shows the CK content on EDL muscles 24 h after perimuscular venom injection. Data report mean ± SEM ($n=5$). *$p < 0.05$ for the difference between treated groups and the venom. Panel A and B Two-Way ANOVA Bonferroni's post-hoc test and Panel C ANOVA Dunnett's post-hoc test.

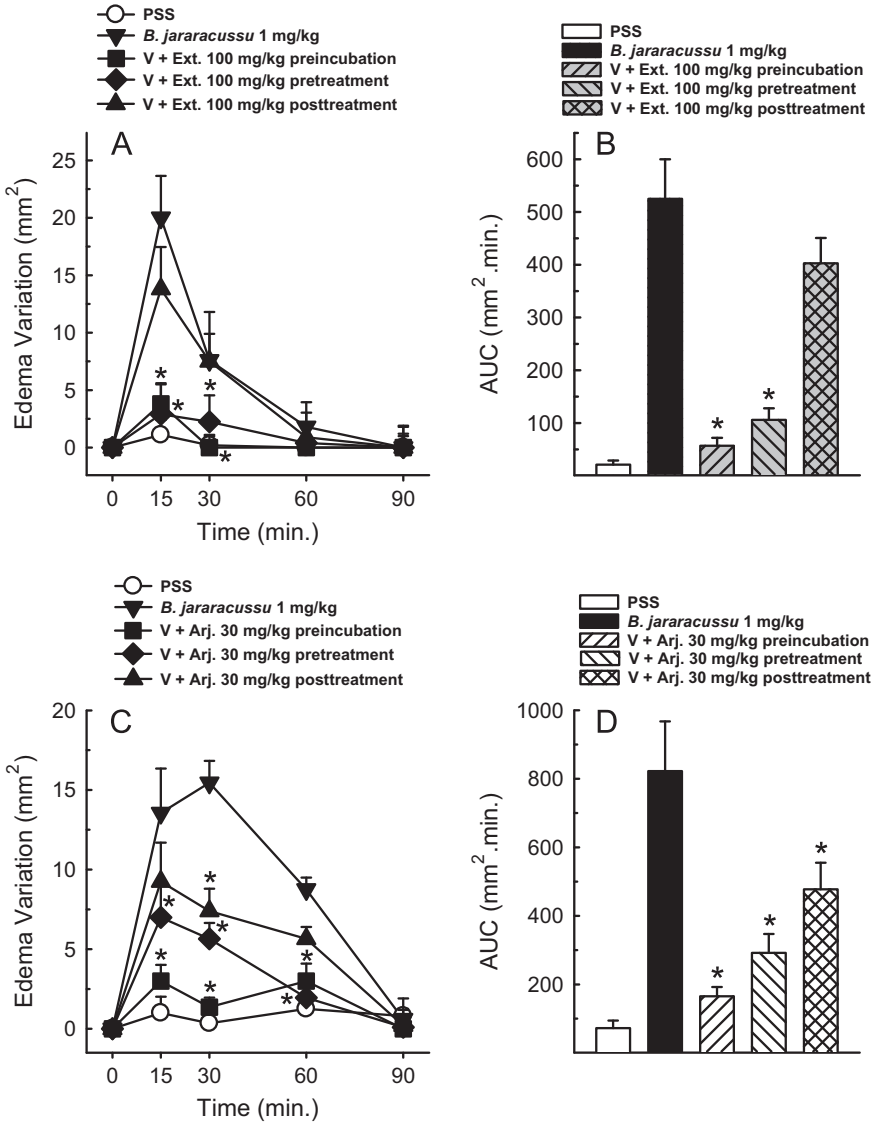


Fig. 4. Effect of *Combretum leprosum* extract and arjunolic acid on *Bothrops jararacussu* venom edematogenic activity. Mice received intramuscular injection (0.05 mL) of PSS or *Bothrops jararacussu* venom (V) alone or associated with *Combretum leprosum* extract (Ext., panels A and B) or arjunolic acid (Arj., panels C and D) in different protocols. Panels A and C show hind limb edema measured with a caliper rule until 90 min. after venom injection, with the resulting areas under the curves (panels B and D, respectively). Data report mean ± SEM ($n=5$). $^{*}p<0.05$ for the difference between treated groups and the venom. Panel A and C Two-Way ANOVA Bonferroni's post-hoc test and panels C and D ANOVA Dunnett's post-hoc test.

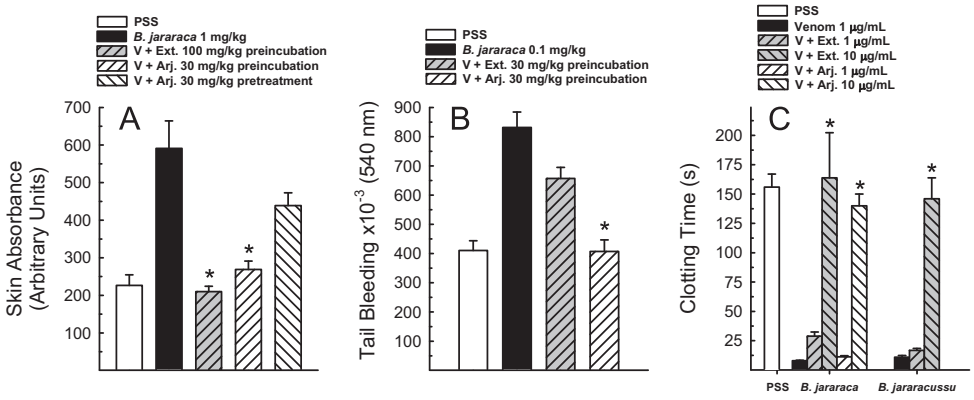


Fig. 5. Effect of *Combretum leprosum* extract and arjunolic acid on *Bothrops jararaca and Bothrops jararacussu* venoms activities on hemostasis. In panel A, mice received intradermic injection (0.1 mL) of physiological saline solution (PSS) or *Bothrops jararaca* venom (V) alone or associated with *Combretum leprosum* extract (Ext.) or arjunolic acid (Arj.) in different protocols. Mice were killed 2 h after venom injection, and the skin was prepared for measurement of absorbance indicating hemorrhagic activity. In Panel B, mice tail bleeding induced by *Bothrops jararaca* venom (V) alone or preincubated with *Combretum leprosum* extract (Ext.) or arjunolic acid (Arj.). Panel C shows the time spent for mice blood collected in a capillary (previously containing venom alone or associated with treatments) to clot. Data report mean ± SEM ($n=5$). ANOVA Dunnett's post-hoc test.

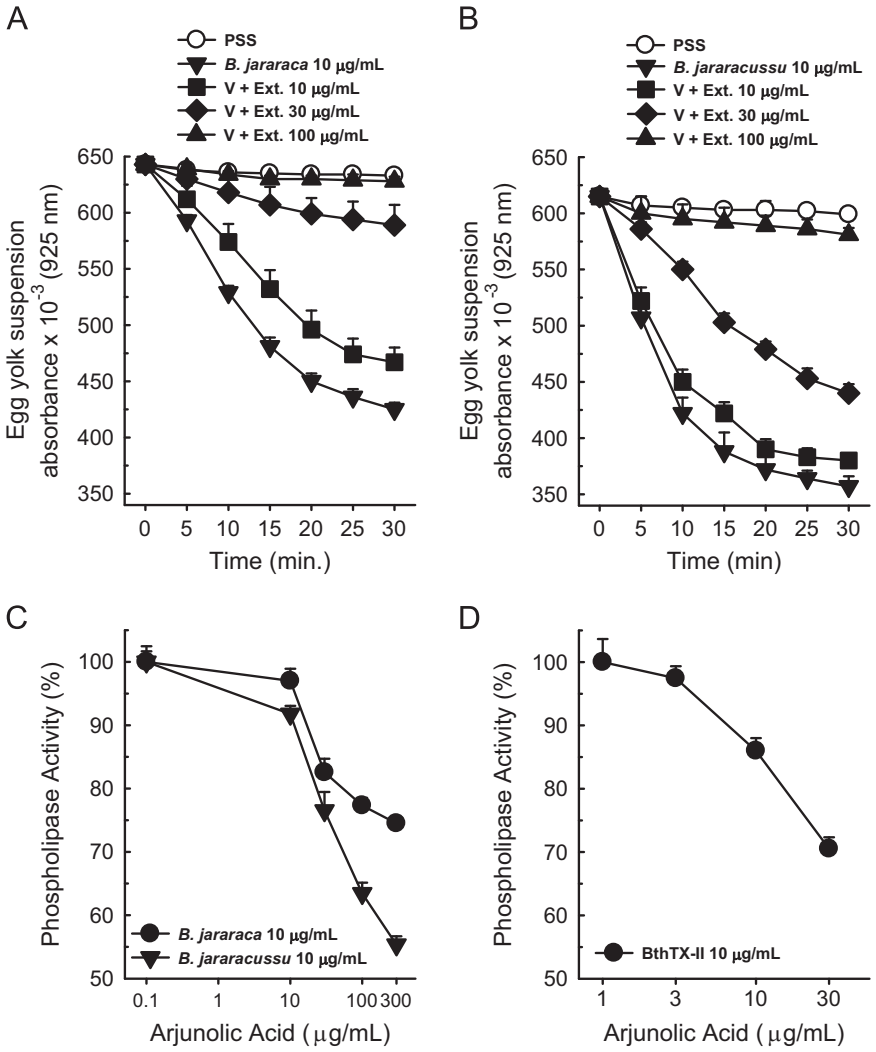


Fig. 6. Effect of *Combretum leprosum* extract and arjunolic acid on *Bothrops jararaca* and *Bothrops jararacussu* venoms phospholipase A_2 activities. Data show the PLA_2 activity of each venom alone (10 μg/mL) or preincubated with increasing concentrations of *Combretum leprosum* extract (*Bothrops jararaca* panel A, *Bothrops jararacussu* panel B). In panels C and D, PLA_2 activities of 10 μg/mL of each venom or bothropstoxin II (BthTx-II) were considered as 100%. Panels show the inhibitory effects of increasing concentrations of arjunolic acid. Data report mean ± SEM ($n=5$).

and time-dependent decrease in turbidity (not shown for BthTx-II), and such activities were considered 100% phospholipase activities. Fig. 6A and B shows the inhibition of phospholipase activities of 10 μg/mL *Bothrops jararacussu* and *Bothrops jararaca* venoms, respectively, by *Combretum leprosum* extract (10–100 μg/mL). On its turn, arjunolic acid also reduced the phospholipase activities of the venoms (6C) and BthTx-II (6D) in a concentration-dependent way. Phospholipase activities were decreased down to approximately 45% and 25% when 300 μg/mL arjunolic acid was incubated with *Bothrops jararacussu* and *Bothrops jararaca* venoms, respectively, and to 30% when 30 μg/mL of the triterpene was incubated with BthTx-II.

3.4.2. Proteolytic activity

Bothrops jararacussu and *Bothrops jararaca* venoms induced hydrolysis of azocasein in a concentration-dependent way (not shown). *Combretum leprosum* extract (0.1–300 μg/mL) decreased, also in a concentration-dependent way, the proteolytic activities of the venoms (10 μg/mL), and the concentration of 300 μg/mL reduced the venoms' proteolytic activities down to zero (Fig. 7A). Arjunolic acid (1–300 μg/mL) also inhibited the hydrolysis of azocasein by the venoms. Inhibition was about 55% and 60% when 300 μg/mL arjunolic acid was incubated with *Bothrops jararacussu* and *Bothrops jararaca* venoms, respectively (Fig. 7B).

3.4.3. Collagenase activity

Bothrops jararacussu and *Bothrops jararaca* venoms induced the release of the Azo group from the substrate (Azocoll®), increasing the absorbance in a concentration-dependent way (data not shown). The incubation of 50 μg/mL of the venoms with *Combretum leprosum* extract (1–300 μg/mL, Fig. 7C) and arjunolic acid (0.1–100 μg/mL, Fig. 7D) antagonized their collagenase activity in a concentration-dependent way. *Combretum leprosum* extract (300 μg/mL) reduced the *Bothrops jararaca* and *Bothrops jararacussu* collagenase activities down to approximately 25 ± 9% and 2 ± 1%, respectively, while arjunolic acid (100 μg/mL) reduced their activities down to 44 ± 9% and to 39 ± 7%, respectively.

3.4.4. Hyaluronidase activity

Bothrops jararacussu and *Bothrops jararaca* venoms degraded hyaluronic acid in a concentration-dependent way (not shown). Arjunolic acid (0.1–30.0 μg/mL) inhibited the enzymatic activity of both venoms. This inhibition was of approximately 65% and 76%

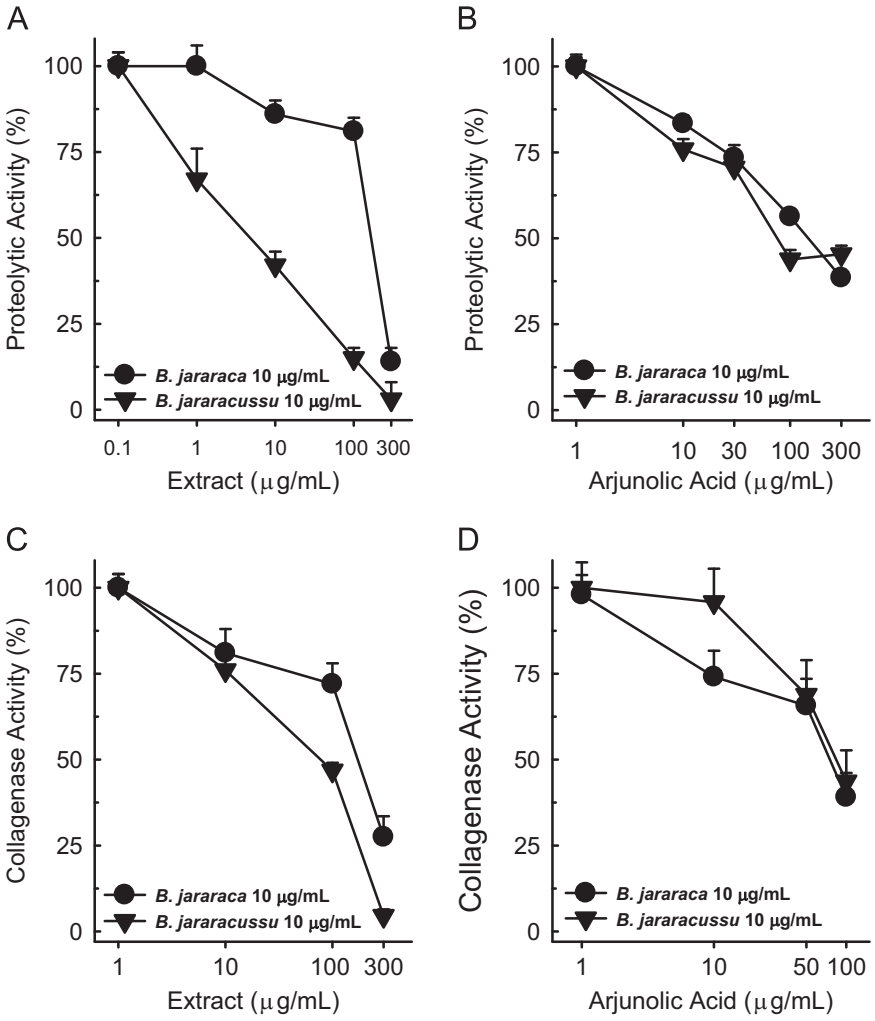


Fig. 7. Effect of *Combretum leprosum* extract and arjunolic acid on *Bothrops jararaca* and *Bothrops jararacussu* venoms proteolytic and collagenase activities. Data show the proteolytic activities of each venom (10 μg/mL) preincubated with increasing concentrations of *Combretum leprosum* extract (panel A) or arjunolic acid (panel B). Accordingly, data on panels C and D show the collagenase activities of each venom (10 μg/mL) preincubated with increasing concentrations of *Combretum leprosum* extract (C) or arjunolic acid (D). The activities of 10 μg/mL of the venoms were considered as 100%. Data report mean ± SEM ($n=5$).

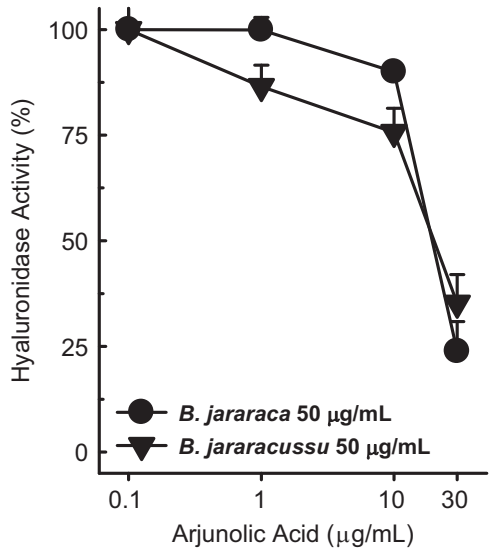


Fig. 8. Effect of arjunolic acid on *Bothrops jararaca* and *Bothrops jararacussu* venoms hyaluronidase activities. Data show the hyaluronidase activities of each venom (50 μg/mL) preincubated with increasing concentrations of arjunolic acid. The activities of 50 μg/mL of the venoms were considered as 100%. Data report mean ± SEM ($n=5$).

when 30 μg/mL arjunolic acid was incubated with *Bothrops jararacussu* and *Bothrops jararaca*, respectively (Fig. 8).

3.5. Inhibition of lethality

Finally, after observing the effects of the venoms on both enzymatic and cellular/tissue levels, we assessed the lethality of *Bothrops jararacussu* venom and its inhibition by arjunolic acid. Intraperitoneal injection of *Bothrops jararacussu* venom induced the death of all the animals in up to 48 h. Oral pre-treatment with 30 and 100 mg/kg arjunolic acid reduced the lethality in more than 65% and 80%, respectively, while preincubation with the triterpene (100 mg/kg) prevented the death of all the animals. Posttreatment, on the other hand, did not prevent the animals' death (data not shown).

4. Discussion

Our results have shown that *Combretum leprosum* extract could inhibit some important toxic activities of the venoms of the snakes *Bothrops jararacussu* and *Bothrops jararaca*, both *in vitro* and *in vivo*. We observed a concentration-dependent protection of the mouse EDL muscle by the extract, *in vitro*, from the myotoxic

effect of *Bothrops jararacussu* venom. This protective effect was also observed in the experiments *in vivo* under different protocols. The cytotoxic effect induced by *Bothrops* venoms is attributed to the presence of complex toxins, which have been targeted by several pharmacological studies showing different degrees of inhibition by different natural and synthetic substances (Melo et al., 1993, 1994; Arruda et al., 2002; Calil-Elias et al., 2002a, 2002b; Sifuentes et al., 2008; Patrão-Neto et al., 2013; Strauch et al., 2013).

In agreement with the present results, the work of de Morais Lima et al. (2012) has shown that some *Combretum* species are extensively used in traditional medicine against inflammation, infections, diabetes, malaria, bleeding, diarrhea and digestive disorders and others as a diuretic. Some studies involving the investigation of biological activities of *Combretum leprosum* point to similar actions as those observed for other species of the genus. Facundo et al. (2005) demonstrated that oral administration of both the root extract of *Combretum leprosum* and arjunolic acid presented anti-inflammatory activity reducing the paw edema induced by carrageenan. Oral arjunolic acid was still capable of reducing ear edema induced by topical application of arachdonic acid (AA), but did not alter the ear edema formed by applying topical 12-O-tetradecanoylphorbol-13-acetate (TPA), suggesting that the compound would be acting to influence the route of metabolism of AA by action of the enzyme cyclooxygenase (COX). Also in this study it was found that the extract of the roots of *Combretum leprosum* was able to inhibit the activity of enzymes butyryl and acetylcholinesterase by action of arjunolic acid. Longhi-Balbinot et al. (2012) and Lopes et al. (2012) demonstrated the antinociceptive activities of 3β, 6β, 16β-trihydroxylup-20(29)-ene (TTHL) and epicatechin, respectively, compounds isolated from extracts of *Combretum leprosum*, which may involve the participation of complex enzymatic systems. Recently, Horinouchi et al. (2013) have also demonstrated antiproliferative and anti-inflammatory properties of flower extract of *Combretum leprosum* in models of skin inflammatory and hyperproliferative process.

On the other hand, the myotoxic effect of *Bothrops jararacussu* venom has been ascribed to the action of its components with proteolytic and phospholipase A_2 activities, as well as to some basic polypeptides that can act directly disrupting the sarcolemma (Queiroz et al., 1984; Homsi-Brandeburgo et al., 1988; Calil-Elias et al., 2002a, 2002b; Gutiérrez and Ownby, 2003; Murakami et al., 2005). Additionally, recent data have shown a strong contribution of inflammation in the process of myonecrosis induced by *Bothrops* venoms (Patrão-Neto et al., 2013). One interesting point has been observed in the experiments carried out with isolated EDL muscle when we added *Combretum leprosum* to the bath solution containing *Bothrops jararacussu* venom. Initially, the high rate of CK release caused by the venom was decreased by the extract, but when we washed the extract out from the solution, exposing the muscle only to the venom, the rate of CK release increased again. This protocol showed that the inhibition was readily reversible, suggesting that the extract and venom must interact for the antagonism to occur.

The *in vivo* protocols showed that the pre-incubation of *Bothrops jararacussu* venom and *Combretum leprosum* extract prevented the increase of plasma CK activity more effectively than the post- and pretreatment protocols. In this last, we tried to reproduce a frequent situation, described in folk medicine, when people take antiophidic plant preparations before any possible contact with snakes (Mors et al., 1989, 2000a; Strauch et al., 2013). Similarly to the specific antivenom, our pretreatment protocols with *Combretum leprosum* extract suggest that protection of the sarcolemma from the venom depends, at least in part, on the direct interaction of venom components with *Combretum leprosum* compounds. These data are in agreement with previous studies showing the same grade of venom neutralization by the antibothropic polyvalent antivenom, by using *E. prostrata* crude extract or its isolated component wedelolactone, as well as some polyanions as antimyotoxic agents (Melo and Suarez-Kurtz, 1988a, 1988b; Melo et al., 1993, 1994; Arruda et al., 2002; Da Silva et al., 2007). This antimyotoxic effect of *Combretum leprosum* extract can be attributed to the inhibition of active enzyme components from the venom by the extracted compounds, as suggested for other active antiophidic agents (Melo et al., 1994; Melo and Ownby, 1999). This hypothesis can be supported by the studies of Facundo et al. (1993, 2005) that have described the presence of different compounds such as triterpenes and flavonoids in *Combretum leprosum*, which may be involved in many biological activities. One of these triterpenes is arjunolic acid, which is an active compound found in different *Combretum* species (Bisoli et al., 2008; Masoko et al., 2008). In our study, arjunolic acid antagonized the myotoxicity of *Bothrops jararacussu* venom both *in vivo* and *in vitro*, suggesting that this isolated compound is able to partially reproduce the crude extract activity. It may, thus, represent the mechanism involved in the antivenom activity.

The acute edema induced by *Bothrops jararacussu* venom was inhibited by *Combretum leprosum* extract and arjunolic acid in different protocols. The edema induced by viperid venoms may be explained by the transitory inflammatory process initiated by cytotoxic venom components and mediators such as autacoids, cyclic endoperoxide, serotonin, histamine, and agents that are not directly inhibited by the polyvalent antivenom (Rojas et al., 2005; Patrão-Neto et al., 2013). The edema antagonism could be the result of the inhibition of such enzymatic venom components. For example, both the extract and arjunolic acid showed the ability to inhibit *Bothrops jararacussu* venom PLA_2 activity, and this action could be related to the reduction of the edema, similarly to other substances that inhibited PLA_2 activity and the inflammatory edema induced by bothropic venoms (Landucci et al., 2000; Marcussi et al., 2007; Santos-Filho et al., 2008; Patrão-Neto et al., 2013). The increased vascular permeability, leading to loss of fluid to the extracellular space (edema), also causes hemoconcentration, as shown by the increased hematocrit values in animals receiving venom injection. Our results indicate hemoconcentration two hours after venom injection, but we believe that the hematocrit values could have been higher before the point of blood collection, probably accompanying the edematogenic activity of the venom. As well as edema inhibition, arjunolic acid also significantly decreased hemoconcentration.

Our experiments have demonstrated a significant antagonism by the crude extract and by arjunolic acid on the hemostatic disturbances induced by *Bothrops jararaca* or *Bothrops jararacussu* venoms. Both bleeding amount *in vivo* and the clotting time *in vitro* were antagonized by the crude extract or by arjunolic acid in a dose- or concentration-dependent manner, respectively. A dose-dependent antagonism was observed in the subcutaneous hemorrhage induced by *Bothrops jararaca* crude venom in the pre-incubation protocol. *Bothrops jararaca* venom has several metalloproteases and coagulation-disturbing proteins that induce local hemorrhage and systemic coagulopathies. The hemorrhagic activity results from the angiorrhexis induced by metalloproteases or collagenase from the venom, which change the constitution of the vascular wall and basal lamina (Kamiguti et al., 1986, 1991; Baramova et al., 1990; Paine et al., 1992; Bjarnason and Fox, 1994; Fujimura et al., 1995). Previous studies have reported that these hemorrhagic proteases are members of the reprolysin family of zinc metalloprotease, induce rapid degradation of sub-endothelial matrix proteins and disruption of the blood vessel, and are involved in local lesions and systemic hemorrhage (Paine et al., 1992; Kawano et al., 2002; Laing and Moura-da-Silva, 2005; Gutiérrez et al., 2005). The *Combretum leprosum* crude extract

inhibited not only the hemorrhagic activity, but also the pro-clotting activity of *Bothrops jararaca* and *Bothrops jararacussu* venoms. These venoms induce a decrease in clotting time that may be directly related to the proteolytic or thrombin-like activities. Previous studies have shown that some venom metalloproteases are "thrombin-like" enzymes that degrade coagulation factors and, moreover, interact with the basal membrane and vascular wall, inducing angiorrhexis and hemorrhage (Mebs and Ownby, 1990; Bjarnason and Fox, 1994; Gutiérrez and Rucavado, 2000). The antagonism of *Bothrops jararacussu* and *Bothrops jararaca* pro-clotting activities *in vitro* may be directly related to the inhibition of "thrombin-like" enzymes from the venom that produce high levels of fibrin, inducing fibrinogen depletion and leading to the hemorrhagic process. In agreement with this hypothesis are the results obtained with the inhibition of different proteolytic enzymes such as caseinolytic enzyme from *Bothrops jararacussu* venom, collagenase from bacteria, and *Bothrops* venom collagenase activities. It is possible that compounds in the *Combretum leprosum* extract inhibit the different enzyme activities and/or the pharmacological sites of action of these toxins (Melo and Ownby, 1999; Murakami et al., 2005). However, the mechanisms of inhibition, caused by the extract and arjunolic acid, still need additional study.

Taken together, our results have shown that *Bothrops jararacussu* and *Bothrops jararaca* venoms presented myotoxic, edematogenic, hemorrhagic, pro-coagulant and diverse enzymatic activities. All these actions, which are characteristics of many viperid venoms, including those of *Bothrops* snakes, may contribute differently for the lethality observed by the intraperitoneal injection of *Bothrops jararacussu* venom, which is assumed to be a consequence of either primary cardiovascular collapse or the collapse secondary to systemic hemorrhage, plasma extravasation and hemoconcentration (increased cardiac workload) (Sifuentes et al., 2008; Gutiérrez et al., 2009, 2013b). In our experiments, the preincubation of arjunolic acid with *Bothrops jararacussu* venom prevented the death of all animals, while oral pretreatment decreased the lethality in more than 80%. Altogether, the neutralization of *Bothrops jararacussu* venom lethality by arjunolic acid could be seen as the sum of the inhibition of each toxic activity. However, despite the fact that the lethality potency assay is well established as the gold standard in assessing the preclinical efficacy of potential antiophidic substances (Gutiérrez et al., 2013b), it is the neutralization itself of the most relevant toxic activities induced by medical-relevant snakes, especially *Bothrops* snakes in Brazil, that must be emphasized, due to the complexity of the venoms, the pathophysiology of envenoming and the clinical and social consequences of snakebites.

5. Conclusion

Overall, the present results, for the first time, give scientific support for the popular use of *Combretum leprosum* extract in the treatment of snakebites in Northeast Brazil. Our data highlight an important component of crude extract of *Combretum leprosum*, arjunolic acid, which can be promising as an antiophidic substance, once it was able to neutralize critical points in the tissue damage process induced by the properties of *Bothrops jararacussu* and *Bothrops jararaca* venom hereby investigated.

References

Agra, M.E., 1996, Plantas da medicina popular dos cariris velhos, Paraíba, Brasil: espécies mais comuns, Associação Plantas do Nordeste, João Pessoa, Paraíba, 112 pp.

Arruda, E.Z., Silva, N.M.V., Moraes, R.A.M., Melo, P.A., 2002. Effect of suramin on myotoxicity of some crotalide snake venoms. Brazilian Journal of Medical Biological Research 35, 723–726.

Baramova, E.N., Shannon, J.D., Bjarnason, J.B., Fox, J.W., 1990. Identification of cleavage sites by a hemorrhagic metalloproteinase in type IV collagen. Matrix 10, 91–97.

Bisoli, E., Garcez, W.S., Hamerski, L., Tieppo, C., Garcez, F.R., 2008. Bioactive pentacyclic triterpenes from the stems of *Combretum laxum*. Molecules 13, 2717–2728.

Bjarnason, J.B., Fox, J.W., 1994. Hemorrhagic metalloproteinases from snake venoms. Pharmacology & Therapeutics 62, 325–372.

Borges, M.H., Soares, A.M., Rodrigues, V.M., Oliveira, F., Fransheschi, A.M., Rucavado, A., Giglio, J.R., Homsi-Brandeburgo, M.I., 2001. Neutralization of proteases from *Bothrops* snake venoms by the aqueous extract from *Casearia sylvestris* (Flacourtiaceae). Toxicon 39, 1863–1869.

Broze Jr., G.J., Yin, Z.F., Lasky, N., 2001. A tail vein bleeding time model and delayed bleeding in hemophiliac mice. Thrombosis and Haemostasis 85, 747–748.

Calil-Elias, S., Martinez, A.M., Melo, P.A., 2002a. Effect of heparin and antivenom on skeletal muscle damage produced by *Bothrops jararacussu* venom. Histology and Histopathology 17, 463–470.

Calil-Elias, S., Thattassery, E., Martinez, A.M., Melo, P.A., 2002b. Effect of perimuscular injection of *Bothrops jararacussu* venom on plasma creatine kinase levels in mice: influence of dose and volume. Brazilian Journal of Medical Biological Research 35, 1233–1235.

Chavira Jr., R., Burnett, T.J., Hageman, J.H., 1984. Assaying proteinases with azocoll. Analytical Biochemistry 136, 446–450.

Chippaux, J.P., Williams, V., White, J., 1991. Snake venom variability: methods of study, results and interpretation. Toxicon 29, 1279–1303.

Chippaux, J.P., Goyffon, M., 1997. Venoms, antivenoms and immunotherapy. Toxicon 36, 823–846.

Coe, F.G., Anderson, G.J., 2005. Screening of medicinal plants used by the Garifuna of eastern Nicaragua for bioactive compounds. Journal of Ethnopharmacology 96, 303.

Da Silva, A.J., Coelho, A.L., Simas, A.B., Moraes, R.A., Pinheiro, D.A., Fernandes, F.F., Arruda, E.Z., Costa, P.R., Melo, P.A., 2004. Synthesis and pharmacological evaluation of prenylated and benzylated pterocarpans against snake venom. Bioorganic & Medicinal Chemistry Letters 14, 431–435.

Da Silva, N.M., Arruda, E.Z., Murakami, Y.L., Moraes, R.A., El-Kik, C.Z., Tomaz, M.A., Fernandes, F.F., Oliveira, C.Z., Soares, A.M., Giglio, J.R., Melo, P.A., 2007. Evaluation of three Brazilian antivenom ability to antagonize myonecrosis and hemorrhage induced by *Bothrops* snake venoms in a mouse model. Toxicon 50, 196–205.

de Morais Lima, G.R., de Sales, I.R., Caldas Filho, M.R., de Jesus, N.Z., de Sousa Falcão, H., Barbosa-Filho, J.M., Cabral, A.G., Souto, A.L., Tavares, J.F., Batista, L.M., 2012. Bioactivities of the genus *Combretum* (Combretaceae): a review. Molecules 17, 9142–9206.

Di Ferrante, N., 1956. Turbidimetric measurement of acid mucopolysaccharides and hyaluronidase activity. Journal of Biological Chemistry 220, 303–306.

Facundo, V.A., Andrade, C.H.S., Silveira, E.R., Braz-Filho, R., Hufford, C.D., 1993. Triterpenes and flavonoids from *Combretum leprosum*. Phytochemistry 32, 411–415.

Facundo, V.A., Rios, K.A., Medeiros, C.M., Militão, J.S.L.T., Miranda, A.L.P., Epifanio, R.A., Carvalho, M.P., Andrade, A.T., Pinto, A.C., Rezende, C.M., 2005. Arjunolic acid in the ethanolic extract of *Combretum leprosum* root and its use as a potential multifunctional phytomedicine and drug for neurodegenerative disorders: anti-inflammatory and anticholinesterasic activities. Journal of the Brazilian Chemical Society 16, 1309–1312.

Fujimura, Y., Ikeda, Y., Miura, S., Suzuki, M., Titani, K., Kawasakl, T., 1995. Isolation and characterization of *B. jararaca* GPIb-BP, a snake venom antagonist specific to platelet glycoprotein Ib. Thrombosis and Haemostasis 74, 743–750.

Garcia, E.S., Guimaraes, J.A., Prado, J.L., 1978. Purification and characterization of a sulfhydryl-dependent protease from *Rhodnius prolixus* midgut. Archives of Biochemistry and Biophysics 188, 315–322.

Gutiérrez, J.M., Rucavado, A., 2000. Snake venom metalloproteinases: their role in the pathogenesis of local tissue damage. Biochimie 82, 841–850.

Gutiérrez, J.M., Ownby, C.L., 2003. Skeletal muscle degeneration induced by venom phospholipases A_2: insights into the mechanisms of local and systemic myotoxicity. Toxicon 42, 915–931.

Gutiérrez, J.M., Rucavado, A., Escalante, T., Díaz, C., 2005. Hemorrhage induced by snake venom metalloproteinases: biochemical and biophysical mechanisms involved in microvessel damage. Toxicon 45, 997–1011.

Gutiérrez, J.M., Escalante, T., Rucavado, A., 2009. Experimental pathophysiology of systemic alterations induced by *Bothrops asper* snake venom. Toxicon 54, 976–987.

Gutiérrez, J.M., Warrell, D.A., Williams, D.J., Jensen., S., Brown, N., Calvete, J.J., Harrison, R.A., 2013a. The need for full integration of snakebite envenoming within a global strategy to combat the neglected tropical diseases: the way forward. PLoS Neglected Tropical Diseases 7, e2162.

Gutiérrez, J.M., Solano, G., Pla, D., Herrera, M., Segura, Á., Villalta, M., Vargas, M., Sanz, L., Lomonte, B., Calvete, J.J., León, G., 2013b. Assessing the preclinical efficacy of antivenoms: from the lethality neutralization assay to antivenomics. Toxicon 69, 168–179.

Havsteen, B., 1983. Flavonoids, a class of natural products of high pharmacological potency. Biochemical Pharmacology 32, 1141–1148.

Homsi-Brandeburgo, M.I., Queiroz, L.S., Santo-Neto, H., Rodrigues-Simioni, L., Giglio, J.R., 1988. Fractionation of *Bothrops jararacussu* snake venom: partial chemical characterization and biological activity of bothropstoxin. Toxicon 26, 615–627.

Horinouchi, C.D., Mendes, D.A., Soley, B.S., Pietrovski, E.F., Facundo, V.A., Santos, A.R., Cabrini, D.A., Otuki, M.F., 2013. *Combretum leprosum* Mart. (Combretaceae): potential as an antiproliferative and anti-inflammatory agent. Journal of Ethnopharmacology 145, 311–319.

Hutchings, A., Scott, A.H., Lewis, G., Cunningham, A., 1996. Zulu Medicinal Plants: An Inventory. University of Natal Press, Pietermaritzburg.

Kamiguti, A.S., Matsunaga, S., Spir, M., Sano-Martins, I.S., Nahas, L., 1986. Alterations of the blood coagulation system after accidental human inoculation by *Bothrops jararaca* venom. Brazilian Journal of Medical and Biological Research 19, 199–204.

Kamiguti, A.S., Cardoso, J.L.C., Theakston, I.S., Sano-Martins, I.S., Hutton, R.A., Rugman, F.P., Warrel, D.A., Hay, C.R.M., 1991. Coagulopathy and haemorrhage victims of *Bothrops jararaca* envenoming in Brazil. Toxicon 29, 961–972.

Kawano, J., Anai, K., Sugiki, M., Yoshida, E., Maruyama, M., 2002. Vascular endothelial cell injury induced by *Bothrops jararaca* venom; non-significance of hemorrhagic metalloproteinase. Toxicon 40, 1553–1562.

Laing, G.D., Moura-da-Silva, A.M., 2005. Jararhagin and its multiple effects on hemostasis. Toxicon 45, 987–996.

Landucci, E.C., De Castro, R.C., Toyama, M., Giglio, J.R., Marangoni, S., De Nucci, G., Antunes, E., 2000. Inflammatory oedema induced by the lys-49 phospholipase A(2) homologue piratoxin-i in the rat and rabbit. Effect of polyanions and p-bromophenacyl bromide. Biochemical Pharmacology 59, 1289–1294.

Longhi-Balbinot, D.T., Lanznaster, D., Baggio, C.H., Silva, M.D., Cabrera, C.H., Facundo, V.A., Santos, A.R., 2012. Anti-inflammatory effect of triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene obtained from *Combretum leprosum* Mart & Eich in mice. Journal of Ethnopharmacology 142, 59–64.

Lopes, L.S., Marques, R.B., Fernandes, H.B., Pereira, S.S., Ayres, M.C., Chaves, M.H., Almeida, F.R., 2012. Mechanisms of the antinociceptive action of (-) epicatechin obtained from the hydroalcoholic fraction of *Combretum leprosum* Mart & Eic in rodents. Journal of Biomedical Science 19, 68.

Marinetti, G.V., 1965. The action of phospholipase A on lipoproteins. Biochimica et Biophysica Acta 98, 554–565.

Marcussi, S., Sant'Ana, C.D., Oliveira, C.Z., Rueda, A.Q., Menaldo, D.L., Beleboni, R.O., Stabeli, R.G., Giglio, J.R., Fontes, M.R., Soares, A.M., 2007. Snake venom phospholipase A2 inhibitors: medicinal chemistry and therapeutic potential. Current Topics in Medicinal Chemistry 7, 743–756.

Masoko, P., Mdee, L.K., Mampuru, L.J., 2008. Biological activity of two related triterpenes isolated from *Combretum nelsonii* (Combretaceae) leaves. Natural Product Research 22, 1074–1084.

McGaw, L.J., Rabe, T., Sparg, S.G., Jäger, A.K., Eloff, J.N., Van Staden, J., 2001. An investigation of the biological activity of *Combretum* species. Journal of Ethnopharmacology 75, 45–50.

Mebs, D., Ownby, C.L., 1990. Myotoxic components of snake venoms: their biochemical and biological activities. Pharmacology & Therapeutics 48, 223–236.

Melo, P.A., Suarez-Kurtz, G., 1988a. Release of sarcoplasmic enzymes from skeletal muscle by *Bothrops jararacussu* venom: antagonism by heparin and by the serum of South American marsupials. Toxicon 26, 87–95.

Melo, P.A., Suarez-Kurtz, G., 1988b. Release of creatine kinase from skeletal muscle by *Bothrops* venoms: heparin potentiation of inhibition by antivenin. Brazilian Journal of Medical and Biological Research 21, 548–558.

Melo, P.A., Homsi-Brandeburgo, M.I., Giglio, J.R., Suarez-Kurtz, G., 1993. Antagonism of myotoxic effects of *Bothrops jararacussu* venom and bothropstoxin by polyanions. Toxicon 31, 285–291.

Melo, P.A., Nascimento, M.C., Mors, W.B., Suarez-Kurtz, G., 1994. Inhibition of the myotoxic and hemorrhagic activities of crotalid venoms by *Eclipta prostrata* (Asteraceae) extracts and constituents. Toxicon 32, 595–603.

Melo, P.A., Ownby, C.L., 1999. Ability of wedelolactone, heparin and para-bromophenacyl bromide to antagonize the myotoxic effects of two crotaline venoms and their PLA_2 myotoxins. Toxicon 37, 199–215.

Mors, W.B., Do Nascimento, M.C., Parente, J.P., Da Silva, M.H., Melo, P.A., Suarez-Kurtz, G., 1989. Neutralization of lethal and myotoxic activities of South American rattlesnake venom by extracts and constituents of the plant *Eclipta prostrata* (Asteraceae). Toxicon 27, 1003–1009.

Mors, W.B., Nascimento, M.C., Pereira, B.M., Pereira, N.A., 2000a. Plant natural products active against snake bite: the molecular approach. Phytochemistry 55, 627–642.

Mors, W.B., Rizzini, C.T., Pereira, N.A., 2000b. Medicinal Plants of Brazil. Reference Publications, Inc., Algonac, MI p. 501.

Murakami, M.T., Arruda, E.Z., Melo, P.A., Martinez, A.M., Calil-Elias, S., Tomaz, M.A., Lomonte, B., Gutiérrez, J.M., Arni, R.K., 2005. Inhibition of myotoxic activity of *Bothrops asper* myotoxin II by the anti-trypanosomal drug suramin. Journal of Molecular Biology 350, 416–426.

Paine, M.J.I., Desmond, H.P., Theakston, R.D., Crampton, J.M., 1992. Purification, cloning, and molecular characterization of a high molecular weight hemorrhagic metalloproteinase, Jararhagin, from *Bothrops jararaca* venom. Journal of Molecular Biology 267, 22869–22876.

Patrão-Neto, F.C., Tomaz, M.A., Strauch, M.A., Monteiro-Machado, M., Rocha Jr., J.R., Borges, P.A., Calil-Elias, S., Melo, P.A., 2013. Dexamethasone antagonizes the *in vivo* myotoxic and inflammatory effects of Bothrops venoms. Toxicon 69, 55–64.

Pio Corrêa, M., 1984. Diccionário de plantas úteis do Brasil e das exóticas cultivadas. Ministério da Agricultura, Brasilia, Instituto Brasileiro de Desenvolvimento Florestal.

Queiroz, L.S., Santo, N.H., Rodrigues-Simioni, L., Prado-Franceschi, J., 1984. Muscle necrosis and regeneration after envenomation by *Bothrops jararacussu* snake venom. Toxicon 22, 339–346.

Raphael, L.S., 1983. Medical Laboratory Technology, 4th ed. Saunders International Edition, 742.

Rojas, E., Quesada, L., Arce, V., Lomonte, B., Rokas, G., Gutierrez, J.M., 2005. Neutralization of four Peruvian *Bothrops* sp. snake venoms by polyvalent antivenoms produced in Peru and Costa Rica: preclinical assessment. Acta Tropica 93, 85–95.

Santos-Filho, N.A., Silveira, L.B., Oliveira, C.Z., Bernardes, C.P., Menaldo, D.L., Fuly, A.L., Arantes, E.C., Sampaio, S.V., Mamede, C.C., Beletti, M.E., de Oliveira, F., Soares, A.M., 2008. A new acidic myotoxic, anti-platelet and prostaglandin I2 inductor phospholipase A2 isolated from *Bothrops moojeni* snake venom. Toxicon 52, 908–917.

Sifuentes, D.N., El-Kik, C.Z., Ricardo, H.D., Tomaz, M.A., Strauch, M.A., Calil-Elias, S., Arruda, E.Z., Schwartz, E.F., Melo, P.A., 2008. Ability of suramin to antagonize the cardiotoxic and some enzymatic activities of *Bothrops jararacussu* venom. Toxicon 51, 28–36.

Strauch, M.A., Tomaz, M.A., Monteiro-Machado, M., Ricardo, H.D., Cons, B.L., Fernandes, F.F., El-Kik, C.Z., Azevedo, M.S., Melo, P.A., 2013. Antiophidic activity of the extract of the Amazon plant Humirianthera ampla and constituents. Journal of Ethnopharmacology 145, 50–58.

Van Wyk, B.E., Van Oudtshoorn, B., Gericke, N., 1997. Medicinal Plants of South Africa. Briza Publications, Pretoria.

Veronese, E.L.G., Esmeraldino, L.E., Trombone, A.P.F., Santana, A.E., Bechara, G.H., Kettelhut, I., Cintra, A.C.O., Giglio, J.R., Sampaio, S.V., 2005. Inihibition of the myotoxic activity of *Bothrops jararacussu* venom and its two major myotoxins, bthTX-I and bthTX-II, by the aqueous extract of *Tabernaemontana catharinensis* A. DC. (Apocynaceae). Phytomedicine 12, 123.

Watt, G., 1989. Snakebite treatment and first aid. In: Campbell, J.A., Lamar, W.W. (Eds.), The Venomous Reptiles of Latin America, pp. 6–13.